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Overexpression of Kinin B_1 Receptors Induces Hypertensive Response to Des-Arg^9-bradykinin and Susceptibility to Inflammation*

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We demonstrated that rat kinin B_1 receptors displayed a ligand-independent constitutive activity, assessed through inositol phosphate production in transiently or stably transfected human embryonic kidney 293A cells. Substitution of Ala for Asn^{130} in the third transmembrane domain resulted in additional constitutive activation of the B_1 receptor. The constitutively active mutant N130A receptor could be further activated by the B_1 receptor agonist des-Arg^9-bradykinin. To gain insights into the physiological function of the B_1 receptor, we have generated transgenic mice overexpressing wild-type and constitutively active mutant receptors under the control of human cytomegalovirus immediately early gene enhancer/promoter. The rat B_1 receptor transgene expression was detected in the aorta, brain, heart, lung, liver, kidney, uterus, and prostate of transgenic mice by reverse transcription-polymerase chain reaction/Southern blot analysis. Transgenic mice were fertile and normotensive. Overexpression of B_1 receptors exacerbated paw edema induced by carrageenan and rendered transgenic mice more susceptible to lipopolysaccharide-induced endotoxic shock. Interestingly, the hemodynamic response to kinins was altered in transgenic mice, with des-Arg^9-bradykinin inducing blood pressure increase when intravenously administered. Our study supports an important role for B_1 receptors in modulating inflammatory responses and for the first time demonstrates that B_1 receptors mediate a hypertensive response to des-Arg^9-bradykinin.

Kinin peptides are released from kininogen precursors by the action of kallikreins in response to tissue injury (1). Kinins induce smooth muscle contraction, vasodilation, increased vascular permeability, and pain (1). Kinins exert their effects through selective activation of two seven-transmembrane domain (TMD)[1] G protein-coupled receptors (GPCRs), B_1 and B_2 (2–4). The B_2 receptor is constitutively expressed, mediating the actions of intact kinins, bradykinin (BK) in rodents and Lys-BK or kallidin in humans (2). In contrast, the B_1 receptor is expressed at very low levels in normal tissues in most animal species but is induced under the influence of inflammation or exposure of tissues to noxious stimuli, mediating the effects of the carboxypeptidase metabolites of intact kinins, des-Arg^9-BK (DABK), and des-Arg^{10}-kallidin (2). The cellular responses of kinin receptors to agonists are transduced primarily via coupling to either G_q protein, which in turn activates phospholipase C to stimulate inositol phosphate production, or the G_i protein, acting through phospholipase A_2 to stimulate arachidonic acid pathway (5, 6).

Over the past years, transgenic and gene-targeting technologies associated with molecular biology tools have provided important knowledge concerning the role of kinin receptors *in vivo*. Transgenic mice expressing the human B_2 receptor under the control of the Rous sarcoma virus 3′-long terminal repeat promoter were hypotensive compared with control littermates (7). Administration of the B_2 receptor antagonist Hoe-140 blunted the blood pressure-lowering effect of the transgene, whereas intra-arterial bolus injection of BK produced more pronounced blood pressure reduction (7). In contrast, deletion of the B_2 receptor in mice produced an unaltered blood pressure phenotype (8) but led to salt-sensitive hypertension and altered nociception (9, 10). Using specific antagonists, the B_1 receptor has been implicated in toxic shock, inflammation, and nociception (11). Studies of mice lacking the B_1 receptor provided support to these observations. B_1 receptor knockout animals were healthy, fertile, and normotensive and exhibited hypoalgesia and reduced inflammatory response (12).

Although much has been learned about the physiological role of the B_1 receptor, most studies are about the lipopolysaccharide (LPS)-induced B_1 receptors, because the B_1 receptor is expressed at very low levels, if at all, in normal tissue. In such experimental set-ups, the animals are under systemic inflammation conditions, which preclude the direct study of the function of the B_1 receptor. Therefore, the precise physiological and pathophysiological roles of the B_1 receptor remain elusive. To extend our understanding of the physiological function of the B_1 receptor, we have created a constitutively active mutant of the rat B_1 receptor and generated transgenic mice that overexpress the wild-type B_1 receptor and the mutant receptor under the control of human cytomegalovirus immediately early gene enhancer/promoter. The transgenic mice were characterized, and the findings are reported here.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney 293A cells were obtained from Quantum Biotechnologies. LPS (*Salmonella enteritidis*, LD_{50}: 7.20 mg/kg) was from Difco Laboratories. *myo*-[^{3}H]Inositol was from PerkinElmer Life Sciences. LipofectAMINE, culture medium, restriction enzymes, and fetal calf serum were bought from Invitrogen. Puro-

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[1] The abbreviations used are: TMD, transmembrane domain; GPCR, G protein-coupled receptor; BK, bradykinin; DABK, des-Arg^9-BK; LPS, lipopolysaccharide; IP, inositol phosphates; MABP, mean arterial blood pressure.

mycin was from Clontech Lab. Hoe-140 was obtained from Hoechst. Sar-Tyr-ϵAhx-Lys-des-Arg^9-bradykinin and Sar-Tyr-ϵAhx-Lys-[D-βNal^7,Ile^8]-des-Arg^9-bradykinin were gifts from Dr. D. Regoli (13). All other chemicals were purchased from Sigma unless stated otherwise.

Site-directed Mutagenesis—The mutations were created by using the QuikChange site-directed mutagenesis kit (Stratagene), and the previously cloned wild-type rat B_1 cDNA in pcDNA3 (Invitrogen) was used as a template (14). The following oligonucleotides were used as forward primers: 5′-GGC CTC TTG GGG GCC CTT TTA GTC TTG TC-3′ for the preparation of clone N54A (nucleotide changes underlined), 5′-GTC ATC AAG GCC GCC CTG TTT GTC AG-3′ for clone N130A, 5′-GCT ATC AGT CAG CAA CGC TAC AGG CTC-3′ for clone D144Q, and 5′-GCT ATC AGT CAG ACC CGC TAC AGG CTC-3′ for clone D144T. The reverse primers were complementary to the forward primers described. All of the mutations were confirmed by DNA sequencing. The *NruI/NotI* fragments in the resultant plasmids were released and inserted at *NruI/NotI* sites in pIRESpuro (Clontech Lab), leading to vectors for stable transfection.

Transfection and Selection—For transient transfection, 293A cells were seeded into 12-well trays and left to adhere overnight. pcDNA3-derived expression vectors were transfected with LipofectAMINE as previously described (14). Transfection mixtures were left on cells for 5 h, and then the cells were treated with a change of standard growth medium for 48 h before functional studies.

For stable transfection, 293A cells were transfected with pIRESpuro-derived expression vectors. After 16 h, the medium was changed with complete medium containing puromycin (2 μg/ml) to start the selection of stably transfected cells. The medium was changed every 3 days, and after about 12 days, the colonies surviving selection were lifted into 12-well plates, expanded with a maintenance concentration of 2 μg/ml puromycin, and screened for ligand specific binding. All of the stock cultures were kept under constant selection pressure of 2 μg/ml puromycin, whereas cells seeded in dishes/wells were maintained without puromycin and used within 2–3 days.

Total Inositol Phosphate Measurement—Monolayers of transfected 293A cells grown in 12-well trays were labeled for 24 h with 2 μCi of [^{3}H]inositol in 0.5 ml of inositol-free Dulbecco's modified Eagles' medium supplemented with 0.05% bovine serum albumin and penicillin/streptomycin. After equilibrated in prewarmed Dulbecco's modified Eagles' medium containing 140 μg/ml bacitracin, 100 μM Captopril, and 25 mM LiCl for 15–30 min, the cells were stimulated with various ligands at the indicated concentrations for 20 min at 37 °C. The released total inositol phosphates (IP) were isolated using Bio-Rad AG1-X8 anion exchange columns (1-ml volume) and quantified as described (15, 16).

Radioligand Binding to Intact Cells—Transfected 293A cell monolayers in 12-well plates were washed twice with Dulbecco's modified Eagles' medium and incubated at 4 °C with radioligand [^{125}I]Sar-Tyr-ϵAhx-Lys-des-Arg^9-bradykinin (14) in the presence or absence of 5 μM of the unlabeled ligand in 0.3 ml of Dulbecco's phosphate-buffered saline supplemented with 140 μg/ml bacitracin, 1 mg/ml bovine serum albumin, 1 mM 1,10-phenanthroline, and 100 μM Captopril. The incubation lasted at least 3 h under gentle agitation. The cells were then rinsed twice with ice-cold phosphate-buffered saline with 0.3% bovine serum albumin followed by solubilization in 0.5 ml of 0.1 M NaOH. The radioactivity of the sample was quantified with a 1261 Mutigamma counter (Pharmacia Corp.). The cell number was determined in parallel wells.

cGMP and cAMP Assays—Stably transfected 293A cells grown in 6-well plates were preincubated for 15 min with Dulbecco's modified Eagles' medium containing 1 mM 3-isobutyl-1-methylxanthine and 100 μM Captopril and then stimulated with 1 μM DABK for 15 min. The stimulation was terminated by exchanging the incubation medium for 0.5 ml of ice-cold 0.1 M HCl. The cGMP and cAMP productions were determined by radioimmunoassys as described (17, 18).

Construction of Wild-type and N130A Mutant Rat B_1 Receptor Transgenes—The bovine growth hormone poly(A) sequence in pcDNA3 was released with *ApaI* and *PvuII* and inserted at *ApaI/Eco*RV sites in pBluescript KS II (Stratagene). The *ApaI/SmaI* bovine growth hormone poly(A) fragment was then released and inserted at *ApaI/SmaI* sites in the pcDNA3-derived wild-type and N130A receptor expression vectors described above. The human 4F2 enhancer was amplified by PCR from genomic DNA with the primers 5′-TAC TCG AGT GCA GCG CGC CCC CG-3′ and 5′-CTG GGC CCT TCA CCT TCA GAG AGC-3′ (19). After being cut with *XbaI* and *ApaI*, the 4F2 enhancer fragment was inserted at *XbaI/ApaI* sites, resulting in final transgene vectors. The vectors were cut with *NruI* and *SmaI*, and the linear transgenes were separated from the unneeded fragments with agarose gel and prepared for injection by Qiaquick gel extraction columns (Qiagen).

Generation of B_1 Receptor Transgenic Mice—Transgenic mice were created by the Transgenic Facility at the Medical University of South Carolina and the Transgenic Facility of University of Ohio at Cincinnati. Linear transgene was injected into the pronuclei of one-cell mouse embryos, which were then surgically implanted into pseudopregnant female mice. Transgenic founder mice were identified by Southern blot analysis of genomic DNA isolated from tail biopsies. Positive founders identified from each line were bred with normal mice, and then F1 littermates were crossed between themselves. Tail DNA was digested with restriction enzyme *KpnI*, run on 0.7% agarose gels containing ethidium bromide, and transferred to Immobilon-N membrane by capillary action with 10× SSC overnight, and the blots were hybridized to a rat kinin B_1 receptor cDNA probe as described previously (14, 20).

Expression of B_1 Receptor Transgene—Total RNA was extracted from mouse tissues using the RNeasy™ columns (Qiagen). Reverse transcription-PCR/Southern blot analysis was performed using the transgene-specific primers and internal probe as previously described (21). The upstream primer is 5′-ATG GCG TCC GAG GTC TT-3′; the downstream primer is 5′-GAC AAA CAC CAG ATC GG-3′; and the internal probe is 5′-TGG CAG CAA CGA CAG AG-3′.

Membrane Preparation and Radioligand Binding Assays—The mice were sacrificed by cervical dislocation. The kidney was removed, and the wet weight was determined. The tissue was homogenized using a Polytron in ~20 volumes of ice-cold 20 mM HEPES, pH 7.4. The membranes from the tissue were prepared, and binding assays were performed as described previously (7, 14).

Blood Pressure Measurements by Tail Cuff—Systolic blood pressure was measured using a computer system RTBP2000 (Kent Scientific) according to the manufacturer's instruction. Briefly, the mice were placed into the prewarmed harness (38 °C). The tail was placed in the occlusion cuff/piezoelectric pulse sensor and distention caused by arterial blood pulses was detected by the sensor and read out onto the computer system. Pressure in the cuff was increased until the pulse was lost. Actual blood pressure was measured as the pressure at which a pulse was detected during cuff depressurization. Ten readings were taken for each animal.

Blood Pressure Measurements by Arterial Cannulation—The mice were initially anesthetized with 2,2,2-tribromoethanol in *tert*-amyl alcohol (Avertin, 20 mg/ml, 0.4 ml/25 g of body weight) and placed on a heated table to maintain body temperature. The right jugular vein and the left carotid artery were cannulated with PE-10 catheters (Clay Adams). After the animals were allowed to recover, blood pressure (in the carotid artery) and heart rate were recorded using a computer system MP100 (Biopac systems Inc). The mice were given a bolus injection of BK or DABK from right jugular vein. BK or DABK was serially diluted and administered at doses of 75, 150, and 300 ng in a volume of 50 μl of saline/mouse.

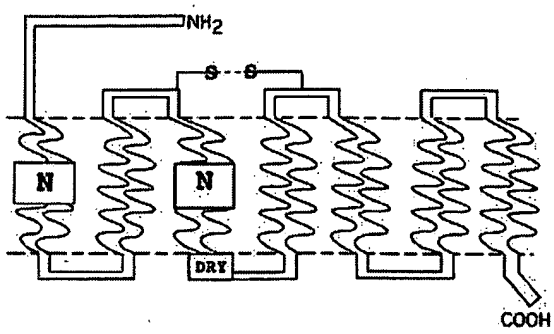
Paw Edema—Inflammation of one hind paw of mice was induced by intraplantar injection of 20 μl of 1% carrageenan (dissolved in saline), 3 μg of capsaicin in 10 μl (dissolved in 5% ethanol, 5% Tween 80 and 90% saline), or DABK (50 or 300 nmol in saline), whereas the contralateral paw received the same volume of vehicle. Thirty min post injection of capsaicin and DABK or 3 h after carrageenan administration, the mice were sacrificed, both hind paws were cut off at the ankle, and the difference between their weights, representing paw edema, was calculated.

Response to Endotoxic Shock—LPS was dissolved in sterile 0.9% NaCl. The mice were injected intraperitoneally with a single dose of LPS (24 mg/kg body weight), and the percentage of survivors was determined at 12-h intervals. Both of the control groups were injected with 0.9% NaCl.

Statistical Analysis—The group data are expressed as the means ± S.E. The data were compared between experimental groups by one-way analysis of variance. Differences between groups were further evaluated by Fisher's protected least squares differences. Differences were considered significant at a value of $p < 0.05$.

RESULTS

Generation and Characterization of Rat B_1 Receptor Mutants—To generate the constitutively active mutants of B_1 receptors, site-directed mutagenesis was directed at the Asn^{54}, Asn^{130}, and Asp^{144} residues in the rat B_1 receptor (Fig. 1). The amino acid replacements were N54A, N130A, D144Q, and D144T. The receptor expression vectors were transiently transfected into 293A cells for assessing constitutive activity by determining agonist-independent IP production. At optimal

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MASEVLLELQPSNRSLQAPANITSCESALEDWDLLYRVL      39
PGEVITICFFGLLGNLLVLSFFLL                     63
PWRQWFWQQRQRQQRLTIAE                         83
IYLANLAASDLVFVLGLPFWAENIG                   108
NRFNWPFGTDLCR                               121
VVSGVIKANLFVSIFLVVAISQ                      143
DRYRLLVYPMTSWGYRRRRQAQ                      165
ATCLLIWVAGGLLSIPTFLL                        185
RSVKVVPDLNVSACILLFPHEAWH                    209
FARMVELNVLGFLLPVTAIIFFNY                    233
HILASLRGQKEASRTRCGGPKGSK                    257
TTGLILTLVASFLVCWCPYHFFAFL                   282
DFLVQVRVIQDCSWKEITD                         301
LGLQLANFFAFVHSCLNPLIYVFAG                   326
RLLKTRVLGTL                                 337
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FIG. 1. *Top panel*, seven-TMD topography of the rat kinin B_1 receptor. *Bottom panel*, amino acids assigned to the different receptor domains. The numbering of the amino acid residues starts with the first N-terminal methionine as 1, and the number refers to the last residue in each line. The seven putative TMDs are *underlined*. The amino acids mutated in the study are indicated with an *asterisk*.

transfection conditions, all of the mutants were expressed at a comparable level but significantly lower than the wild-type receptor (data not shown). As shown in Table I, the wild-type B_1 receptor displayed a marked ligand-independent, spontaneous activity (104% above control levels of the mock-transfected cells), and the N54A mutant showed an impaired basal activity compared with the wild-type receptor. Substitution of Ala for Asn^{130} resulted in significantly constitutive activation of the rat B_1 receptor (409% above control levels). In contrast, the agonist-independent IP accumulations in 293A cells expressing D144Q and D144T mutants were similar to that of mock transfected cells, and the constitutive activity of the wild-type receptor was abolished by the mutations. Interestingly, the maximal extent of DABK stimulation of IP production was significantly reduced for all mutants compared with the wild-type receptor.

To better characterize the N130A mutant receptor, stable expression of the N130A mutant in 293A cells was established. For comparison, stable expression of the wild-type rat B_1 receptor in 293A cells was also established. Such stably transfected 293A cells were analyzed for receptor density and their affinity for radioligand [^{125}I]Sar-Tyr-ϵAhx-Lys-des-Arg^9-bradykinin and used in functional assays. The N130A receptor was expressed at a level of about 25% of the wild-type receptor in the stably transfected 293A cells (Table II). The maximum number of N130A receptor-binding sites (B_{max}) using [^{125}I]Sar-Tyr-ϵAhx-Lys-des-Arg^9-bradykinin is 1.4×10^5 sites/cell *versus* 5.3×10^5 sites/cell for the wild-type receptor. In contrast, the mutation of Asn^{130} into Ala significantly increased the affinity for [^{125}I]Sar-Tyr-ϵAhx-Lys-des-Arg^9-bradykinin. The dissociation constant (K_d) for the binding of [^{125}I]Sar-Tyr-ϵAhx-Lys-des-Arg^9-bradykinin by the wild-type

TABLE I

IP accumulation in 293A cells expressing wild-type or mutant receptors

R_{max} indicates the percentage increase in the 1 μM DABK-induced IP accumulation above basal levels in the absence of DABK. Basal IP indicates the percentage of increase in IP concentrations over those of mock-transfected cells (control) in the absence of DABK. The expression levels of the receptors were comparable except for the wild type (WT) with a higher level of expression. The results are the means ± S.E. of three independent experiments, each performed in duplicate.

Receptor	[^{3}H]IP accumulation	
	R_{max}[a]	Basal IP[b]
WT	1487 ± 112	104 ± 27
N54A	433 ± 35	10 ± 4
N130A	430 ± 18	409 ± 23
D144Q	67 ± 7	0
D144T	20 ± 9	0

[a] The R_{max} values are percentages above basal.
[b] The basal IP values are percentages above control.

and N130A receptors in intact stably transfected 293A cells were 2.54 ± 0.40 and 1.63 ± 0.18 nM, respectively.

To assess the mode of coupling between the B_1 receptor and adenylate cyclase and guanylate cyclase and the effect of the mutation of Asn^{130} into Ala on the DABK-induced cAMP and cGMP production, the intracellular cAMP and cGMP levels were measured in the stably transfected 293A cells. As shown in Table II, the basal cAMP and cGMP levels in the 293A cells stably expressing the wild-type receptor were similar to those in the nontransfected 293A cells, whereas in the 293A cells stably expressing the N130A receptor, the basal cAMP and cGMP levels were elevated. DABK challenge increased intracellular cAMP and cGMP production in the 293A cells stably expressing the wild-type and N130A receptors: 116-fold increase in cAMP levels and 5.2-fold increase in cGMP levels for the wild-type receptor *versus* 14-fold increase in cAMP levels and 1.7-fold increase in cGMP levels for the N130A mutant.

Using the stably transfected 293A cells, the dose-dependent DABK stimulation of IP production for the wild-type and N130A receptor was investigated. As shown in Fig. 2, the N130A mutant could be further activated by over 5-fold by saturation doses of DABK, whereas the wild-type receptor could be amplified to a even higher degree (~16-fold).

The properties of some kinins to modulate IP production by the wild-type and N130A receptors were evaluated. As shown in Fig. 3, kinin B_2 receptor agonist BK and antagonist Hoe-140 have no effects. Des-Arg^9,[Leu^8]-bradykinin and Sar-Tyr-ϵAhx-Lys-[D-βNal^7,Ile^8]-des-Arg^9-bradykinin are human B_1 receptor-specific antagonists. Sar-Tyr-ϵAhx-Lys-[D-βNal^7,Ile^8]-des-Arg^9-bradykinin is still an antagonist for the wild-type rat B_1 receptor, whereas des-Arg^9,[Leu^8]-braykinin becomes a partial agonist (70–80% of DABK), which provides support for the early observation that des-Arg^9,[Leu^8]-bradykinin has partial agonist activity in a contraction assay of smooth muscle of rat duodenum and ileum (22, 23). In contrast, Sar-Tyr-ϵAhx-Lys-[D-βNal^7,Ile^8]-des-Arg^9-bradykinin becomes a partial agonist, and des-Arg^9,[Leu^8]-BK becomes a potent agonist for the N130A receptor.

Generation of Transgenic Mice—Using the wild-type and N130A cDNAs, we have constructed two transgenes for development of transgenic mice. The transgene consists of the cytomegalovirus immediately early gene enhancer/promoter, the wild-type or N130A rat B_1 receptor cDNA, the human 4F2 enhancer, and the bovine growth hormone poly(A) sequence (Fig. 4). Three transmitting founder lines, including one wild-type line, WT2510, and two N130A lines, N130A58 and N130A2592, were identified by Southern blot analysis of genomic DNA. Heterozygous wild-type and N130A transgenic

TABLE II
Characterization of the wild-type and N130A mutant receptors

293A cells stably expressing the wild-type (WT) and the N130A mutant (N130A) rat kinin B_1 receptor were established, and the receptor binding sites (B_{max}) and dissociation constant (K_d) were determined by using radioligand [^{125}I]Sar-Tyr-εAhx-Lys-des-Arg^9-bradykinin as described under "Experimental Procedures." The basal (Basal) and 1 μM DABK-stimulated (Maximal) intracellular cAMP and cGMP levels were also measured. The results are the means ± S.E. of three experiments.

Receptor	K_d	B_{max}	cAMP		cGMP	
			Basal	Maximal	Basal	Maximal
	nM	*(sites/cell × 10^{-3})*	*pmol/mg*		*fmol/mg*	
WT	2.54 ± 0.40	530	16 ± 2	1858 ± 55	421 ± 29	2208 ± 218
N130A	1.63 ± 0.18	140	43 ± 7	608 ± 53	528 ± 21	872 ± 14
293A cells			12 ± 1	13 ± 5	454 ± 14	461 ± 35

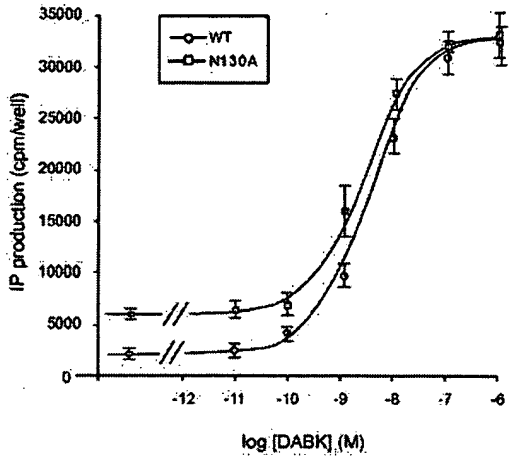

FIG. 2. **Dose-dependent DABK stimulation of IP production by the wild-type and N130A mutant B_1 receptors.** The stimulation of IP production by various concentrations of DABK was analyzed in the 293A cells stably expressing the wild-type (*WT*) or N130A mutant (*N130A*) receptors (expression levels = 5.3 × 10^5 and 1.4 × 10^5 sites/cell, respectively) as described under "Experimental Procedures." The results are representative of three independent experiments, each performed in duplicate.

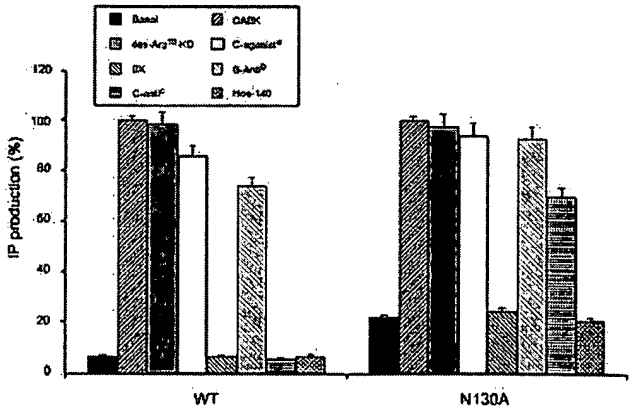

FIG. 3. **Effects of various kinins on IP production by the wild-type and N130A mutant B_1 receptors.** The effects of kinin peptides (100 nM) on IP production were analyzed in the 293A cells stably expressing the wild-type (*WT*) or N130A mutant (*N130A*) receptors (expression levels = 5.3 × 10^5 and 1.4 × 10^5 sites/cell, respectively) as described under "Experimental Procedures." IP production by various ligand stimulations was expressed as the percentage of IP production by DABK (100 nM) stimulation for the wild-type and N130A receptors, respectively. The results are representative of three independent experiments, each performed in duplicate. *a*, Sar-Tyr-εAhx-Lys-des-Arg^9-BK; *b*, des-Arg^9,[Leu^8]-BK; *c*, Sar-Tyr-εAhx-Lys-[D-βNal^7,Ile^8]-des-Arg^9-BK.

mice showed no gross phenotypic abnormalities. All of the transgenic mice were fertile. However, mating F1 generation heterozygotes of both N130A lines produced smaller litters

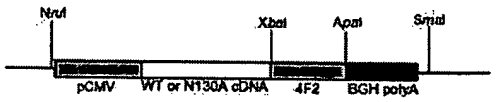

FIG. 4. **Rat kinin B_1 receptor transgene constructs.** The *open bar* represents wild-type (*WT*) or mutant N130A rat kinin B_1 receptor cDNA. *pCMV* denotes the human cytomegalovirus immediately early gene enhancer/promoter, *4F2* represents the human 4F2 heavy-chain gene enhancer, and *BGH polyA* denotes the bovine growth hormone gene polyadenylation sequence.

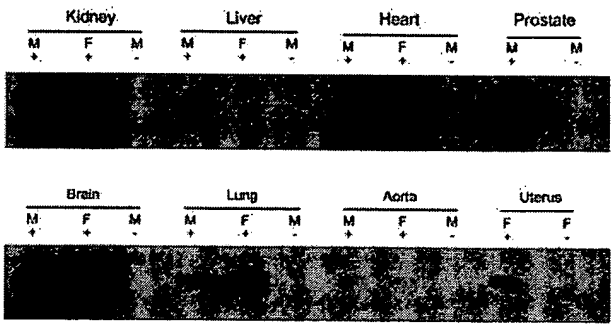

FIG. 5. **Expression of N130A mutant mRNA in transgenic mice.** Reverse transcription-PCR Southern blot revealed the expression of mutant N130A receptor mRNA in mouse tissues. 2 μg of total RNA was reversely transcribed, and one-twentieth of the resultant cDNA was then subjected to PCR for 30 cycles, followed by Southern blot analysis. + represents N130A58 mice, and − represents nontransgenic mice. *F* indicates female, and *M* indicates male.

compared with the nontransgenic control and WT2510 line, and a non-Mendelian ratio against N130A mice was observed in the offspring. In the following studies heterozygous transgenic mice were used.

Expression of B_1 Receptor Transgene—The distribution of transgene mRNA expression in F1 and F2 generation heterozygous mice was determined by reverse transcription-PCR/Southern blot analysis. As expected, both male and female transgenic mice showed significant overexpression of the transgene mRNA in the aorta, kidney, liver, heart, brain, and lung and in the prostate of males and the uterus of females. Fig. 5 shows the result from line N130A58. Using specific B_1 receptor radioligand [^{125}I]Sar-Tyr-εAhx-Lys-des-Arg^9-BK (14), strong B_1 receptor binding activity was detected in membranes prepared from the kidneys of all transgenic lines (data not shown). In contrast, neither transgene mRNA expression nor B_1 receptor (including endogenous) binding activity could be detected in the corresponding tissues of the nontransgenic control mice.

Blood Pressure—All of the heterozygous transgenic mice were normotensive. The systolic blood pressures of transgenic mice 10 weeks old were 78.7 ± 8.5 mmHg (n = 11) for line WT2510, 75.0 ± 7.6 mmHg (n = 14) for line N130A58, and 76.2 ± 6.3 mmHg (n = 11) for line N130A2592 *versus* 80.9 ± 3.9 mmHg (n = 11) for age-matched nontransgenic control littermates. Intravenous injection of B_1 receptor agonist DABK via

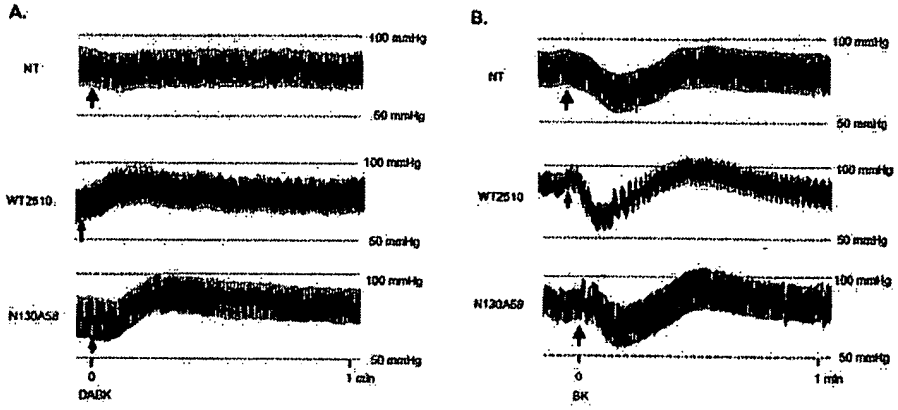

FIG. 6. **Effects of DABK and BK on the MABP of mice.** Typical tracing of the blood pressure response to 75 ng of DABK (*A*) or 300 ng of BK (*B*) in anesthetized mice is shown. Kinins were injected intravenously at time 0. *NT*, nontransgenic mice.

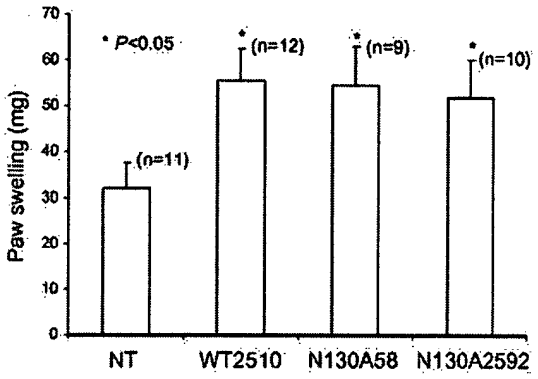

FIG. 7. **Carrageenan-induced paw edema in mice.** Transgenic and nontransgenic control mice received intraplantar injection of the carrageenan suspension into one hind paw and received the vehicle into the other paw. After 3 h, the animals were sacrificed, both hind paws were cut off at the ankle, and the difference between their weights was determined. *NT*, nontransgenic mice. (For nontransgenic mice *versus* three transgenic lines, $p < 0.05$.)

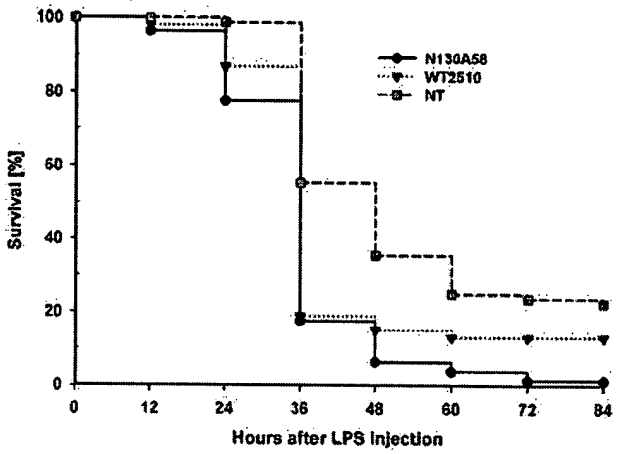

FIG. 8. **Survival rate of mice after endotoxic shock.** Transgenic and their nontransgenic control mice were subjected to LPS injection (24 mg/kg, intraperitoneally). The percentage of surviving mice was determined at 12-h intervals. (For line WT2510, $n = 53$; for line N130A58, $n = 80$; for nontransgenic mice (*NT*), $n = 76$.)

the jugular vein produced a transient increase of mean arterial blood pressure (MABP) in anesthetized transgenic mice but not in nontransgenic control littermates (Fig. 6*A*). The duration of MABP increase lasted over 5 min. 75 ng of DABK led to an increase of blood pressure by up to 15 mmHg. In contrast, intravenous injection of B_2 receptor agonist BK into transgenic mice caused a remarkable primary MABP reduction, followed by a blood pressure bounce-back going beyond basal level, whereas in nontransgenic mice the blood pressure just returned to basal level, after a similar primary MABP reduction (Fig. 6*B*). Single or subsequent injections of higher doses of DABK did not result in a further increase of blood pressure in transgenic mice (data not shown).

Inflammation—Intraplantar injection of carrageenan resulted in a marked inflammation seen by paw swelling in normal and transgenic mice (Fig. 7). But the paw edema induced in transgenic mice was more severe. The percentage of the weight increase of the carrageenan-injected paw over the contralateral vehicle-injected paw was 38.4 ± 6.7% for line WT2510, 42.1 ± 5.0% for line N130A58, and 37.4 ± 6.5% for line N130A2592 *versus* only 21.7 ± 5.8% for nontransgenic control mice. However, there was no significant difference in paw weight increase between transgenic mice and nontransgenic mice after induction by either capsaicin or DABK (data not shown). We then evaluated the response of transgenic mice to the lethal effects of endotoxic shock. To this end, the mice were injected with a high dose of LPS (24 mg/kg of body weight). Fig. 8 shows the percentage of survivors after LPS injection. Within the first 36 h about 82% of transgenic mice from lines WT2510 and N130A58, but only 45% of nontransgenic mice died. After 3 days, the mortality rate of WT2510 and N130A58 mice reached 87 and 99%, respectively, whereas nontransgenic mice reached only 76%. In contrast, mock injection of vehicle did not cause death in either group.

DISCUSSION

In the absence of agonist, GPCRs spontaneously isomerize between the inactive and active conformations, with the equilibrium shifted toward the predominantly inactive conformation (24, 25). Some receptor mutations induce an agonist-independent shift in isomerization equilibrium toward the active conformation and evoke second messenger responses in the absence of agonist. Such constitutive activations of GPCRs are generally believed to result from an increase in receptor conformational flexibility caused by the loss of intramolecular constraints (26). Our present study shows that the wild-type rat kinin B_1 receptor displayed a marked constitutive activity in the transiently or stably transfected 293A cells. During the preparation of this manuscript, Leeb-Lundberg *et al.* (27) reported that the human counterpart B_1 receptor also exhibited a high level of constitutive activity in transiently transfected 293 cells and so did the rabbit wild-type B_1 receptor, suggesting that a high constitutive activity might be a common characteristic of the B_1 receptor.

Using single amino acid replacements based on findings from other GPCRs, we generated four mutants of rat B_1 receptors. Asn^{54} in TMD I is highly conserved in the superfamily of

seven-TMD GPCRs (28). It was thought to be part of a highly conserved transmembrane "polar pocket," involved in receptor activation (29). Substitution of the homologous Asn residue with Ala induced a moderate constitutive activation of the α_{1B}-adrenergic receptor (29). However, in the case of the rat B_1 receptor, similar mutation did not result in enhancement of constitutive activation. Asp^{144} is part of the highly conserved DRY motif (triplet of amino acids: Asp-Arg-Tyr) located at the boundary of TMD III and the second intracellular loop (Fig. 1). The DRY motif has played a pivotal role in the signal transduction pathway of GPCRs (30–32). Mutations of the aspartate residue are reported to lead to constitutive activity for some GPCRs (31–33). Similar mutation in the homologous position in the rat B_1 receptor failed to induce detectable constitutive activity. However, this result is not unprecedented. For some adrenergic and muscarinic receptors, mutations in the aspartate residue of the DRY motif did not result in agonist-independent constitutive activity (34, 35). Asn^{130} in the rat B_1 receptor is 14 residues N-terminal to the DRY motif. Mutations in this homologous position such as Cys^{128} in the α_{1B}-adrenergic receptor (36), Cys^{116} in the β_2-adrenergic receptor (37), and Asn^{111} in the angiotensin AT_1 receptor (38) led to constitutive activity, suggesting that this particular amino acid position may function as a switch that regulates transition between distinct receptor conformations (37). Based on molecular modeling of the AT_1 receptor and their finding that mutation of Asn^{111} to Ala led to constitutive activation of the AT_1 receptor, Bonnafous and co-workers (38) proposed that Asp^{74}, Asn^{111}, and Trp^{253} were involved in the AT_1 receptor activation. They found that these residues were conserved in the kinin B_2 receptor and that mutation of Asn^{113} to Ala in the homologous position resulted in a high constitutive activation of the human kinin B_2 receptor (39). Interestingly, these residues were also conserved in the B_1 receptor (*i.e.* Asp^{93}, Asn^{130}, and Trp^{273} for the rat B_1 receptor). Here we show that mutation of the homologous Asn^{130} to Ala in the rat B_1 receptor induced a marked constitutive activation. Leeb-Lundberg *et al.* (27) reported that mutation of homologous Asn^{121} to Ala in the human B_1 receptor also caused a further increase in constitutive activity, indicating that this Asn is indeed involved in constraining the B_1 receptor in an inactive state. Our findings suggest that the molecular events associated to their activation processes are probably conserved between kinin B_1 and B_2 receptors.

The basal IP production by the N130A receptor in the stably transfected 293A cells was about 3-fold higher than that by the wild-type receptor in the stably transfected 293A cells. But if one considers that the expression level of the N130A receptor is about 25% of that of the wild-type B_1 receptor and that increasing the expression of receptors could increase signal to phospholipase C as demonstrated by other researchers with other GPCRs (27, 31, 39), the N130A receptor would have a up to 12-fold increase in basal activity compared with the wild-type receptor.

Constitutively active mutants have been shown to be responsible for several hereditary and acquired diseases and have been used to produce transgenic mice serving as unique experimental models (40). Because the wild-type rat B_1 receptor has a high basal activity and the N130A mutant is highly constitutively active, we expect that overexpressing these receptors in mice would allow the emergence of any pathophysiological consequences associated with B_1 receptors. B_1 receptor-mediated hypotensive responses have been documented in rabbit, rat, pig, and dog (11). Unexpectedly, we found that all three B_1 receptor transgenic lines were normotensive. One explanation for this finding is that some strong unknown compensatory *in vivo* mechanisms exist and that any such compensation might more likely dilute the manifestation of altered blood pressure phenotype. Alternatively, the B_1 receptor may not be as important in the normal modulation of hemodynamics, which is compatible with the observation that B_1-deficient mice are normotensive (12). It should be noted that the B_1 receptor-mediated hypotensive effect is generally only observed following an inflammatory stimulus, such as endotoxin treatment (11).

Surprisingly, intravenous administration of DABK produced a MABP increase in transgenic mice, and a subsequent injection of DABK was almost as potent as the first injection, which can be explained by the lack of desensitization of B_1 receptors (41). Intravenous injection of BK initially caused blood pressure reduction in transgenic mice, and then the blood pressure bounced back and went beyond the basal level, whereas in the control littermates BK only produced a transient blood pressure reduction. This discrepancy could be due to the conversion of some of the injected BK by kininase I into DABK *in vivo* (42), which then acted on the constitutively expressed B_1 receptor, causing a blood pressure increase in transgenic mice. B_1 receptor ligands have been reported to cause vasoconstriction of a range of blood vessels from several species (43). The DABK-mediated hypertensive effect in B_1 receptor transgenic mice probably resulted from B_1 receptor-induced peripheral resistance. However, the central role of B_1 receptors in hypertension cannot be ruled out at this time. Alvarez *et al.* (44) reported that brain B_1 receptor blockade lowers blood pressure in spontaneously hypertensive rats but not in normotensive rats. Similarly, we demonstrated that intracerebroventricular administration of B_1 receptor agonists increases blood pressure in both Wistar Kyoto rats and spontaneously hypertensive rats, whereas B_1 receptor blockade with antisense oligonucleotides reduced blood pressure in spontaneously hypertensive rats but not in Wistar Kyoto rats (45).

In B_1 receptor transgenic mice, we found that carrageenan-induced paw edema was significantly enhanced compared with that in nontransgenic control littermates. It was reported that carrageenan, a water-extractable polysaccharide obtained from various seaweeds, could activate kinin release and induced the B_1 receptor (46, 47). This enhancement in B_1 receptor transgenic mice is probably because released kinins could immediately activate the constitutively expressed B_1 receptors without time delay for the B_1 receptor induction as in nontransgenic control. Overexpression of B_1 receptors appears to have no significant effects on DABK or capsaicin-induced acute edema. However, this finding is in line with the observation that the acute edema produced by kinins injection into rat paw showed mediation by a form of B_2 receptor, without a significant involvement of B_1 receptors (48), even if the animal was pretreated with LPS (49). Compared with nontransgenic control mice, line WT2510 was prone to endotoxic shock, but N130A lines were more susceptible. The reason is not clear for the time being. But these findings clearly support the notion that B_1 receptors play an important role in modulating inflammatory responses.

REFERENCES

1. Bhoola, K. D., Figueroa, C. D., and Worthy, K. (1992) *Pharmacol. Rev.* **44**, 1–80
2. Regoli, D., and Barabe, J. (1980) *Pharmacol. Rev.* **32**, 1–46
3. Hess, J. F., Borkowski, J. A., Young, G. S., Strader, C. D., and Ransom, R. W. (1992) *Biochem. Biophys. Res. Commun.* **184**, 260–268
4. Menke, J. G., Borkowski, J. A., Bierilo, K. K., MacNeil, T., Derrick, A. W., Schneck, K. A., Ransom, R. W., Strader, C. D., Linemeyer, D. L., and Hess, J. F. (1994) *J. Biol. Chem.* **269**, 21583–21586
5. Schneck, K. A., Hess, J. F., Stonesifer, G. Y., and Ransom, R. W. (1994) *Eur. J. Pharmacol.* **266**, 277–282
6. Tropea, M. M., Gummelt, D., Herzig, M. S., and Leeb-Lundberg, L. M. (1993) *J. Pharmacol. Exp. Ther.* **264**, 930–937
7. Wang, D. Z., Chao, L., and Chao, J. (1997) *Hypertension* **29**, 488–493
8. Borkowski, J. A., Ransom, R. W., Seabrook, G. R., Trumbauer, M., Chen, H., Hill, R. G., Strader, C. D., and Hess, J. F. (1995) *J. Biol. Chem.* **270**,

13706–13710
9. Rupniak, N. M., Boyce, S., Webb, J. K., Williams, A. R., Carlson, E. J., Hill, R. G., Borkowski, J. A., and Hess, J. F. (1997) *Pain* **71**, 89–97
10. Madeddu, P., Varoni, M. V., Palomba, D., Emanueli, C., Demontis, M. P., Glorioso, N., Dessi-Fulgheri, P., Sarzani, R., and Anania, V. (1997) *Circulation* **96**, 3570–3578
11. Marceau, F., Hess, J. F., and Bachvarov, D. R. (1998) *Pharmacol. Rev.* **50**, 357–386
12. Pesquero, J. B., Araujo, R. C., Heppenstall, P. A., Stucky, C. L., Silva, J. A., Jr., Walther, T., Oliveira, S. M., Pesquero, J. L., Paiva, A. C., Calixto, J. B., Lewin, G. R., and Bader, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8140–8145
13. Gobeil, F., Neugebauer, W., Nguyen-Le, X. K., Nea Allogho, S., Pheng, L. H., Blouin, D., Whalley, E. T., and Regoli, D. (1997) *Can. J. Physiol. Pharmacol.* **75**, 591–595
14. Ni, A., Chai, K. X., Chao, L., and Chao, J. (1998) *Biochim. Biophys. Acta* **1442**, 177–185
15. Berridge, M. J., Downes, C. P., and Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
16. Faussner, A., Proud, D., Towns, M., and Bathon, J. M. (1998) *J. Biol. Chem.* **273**, 2617–2623
17. Brooker, G., Harper, J. F., Terasaki, W. L., and Moylan, R. D. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 1–33
18. Wang, D., Yoshida, H., Song, Q., Chao, L., and Chao, J. (2000) *Am. J. Physiol.* **278**, F484–F491
19. Karpinski, B. A., Yang, L. H., Cacheris, P., Morle, G. D., and Leiden, J. M. (1989) *Mol. Cell. Biol.* **9**, 2588–2597
20. Chai, K. X., Ni, A., Wang, D., Ward, D. C., Chao, J., and Chao, L. (1996) *Genomics* **31**, 51–57
21. Ni, A., Chao, L., and Chao, J. (1998) *J. Biol. Chem.* **273**, 2784–2791
22. Paiva, A. C., Paiva, T. B., Pereira, C. C., and Shimuta, S. I. (1989) *Br. J. Pharmacol.* **98**, 206–210
23. Meini, S., Lecci, A., and Maggi, C. A. (1996) *Br. J. Pharmacol.* **117**, 1619–1624
24. De Lean, A., Stadel, J. M., and Lefkowitz, R. J. (1980) *J. Biol. Chem.* **255**, 7108–7117
25. Kenakin, T. (1996) *Pharmacol. Rev.* **48**, 413–463
26. Cotecchia, S., Exum, S., Caron, M. G., and Lefkowitz, R. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2896–2900
27. Leeb-Lundberg, L. M., Kang, D. S., Lamb, M. E., and Fathy, D. B. (2001) *J. Biol. Chem.* **276**, 8785–8792
28. Baldwin, J. M., Schertler, G. F., and Unger, V. M. (1997) *J. Mol. Biol.* **272**, 144–164
29. Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G., and Cotecchia, S. (1996) *EMBO J.* **15**, 3566–3578
30. Oliveira, L., Paiva, A. C., Sander, C., and Vriend, G. (1994) *Trends Pharmacol. Sci.* **15**, 170–172
31. Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G., and Cotecchia, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 808–813
32. Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999) *Mol. Pharmacol.* **56**, 175–184
33. Morin, D., Cotte, N., Balestre, M. N., Mouillac, B., Manning, M., Breton, C., and Barberis, C. (1998) *FEBS Lett.* **441**, 470–475
34. Fraser, C. M., Chung, F. Z., Wang, C. D., and Venter, J. C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5478–5482
35. Savarese, T. M., and Fraser, C. M. (1992) *Biochem. J.* **283**, 1–19
36. Perez, D. M., Hwa, J., Gaivin, R., Mathur, M., Brown, F., and Graham, R. M. (1996) *Mol. Pharmacol.* **49**, 112–122
37. Zuscik, M. J., Porter, J. E., Gaivin, R., and Perez, D. M. (1998) *J. Biol. Chem.* **273**, 3401–3407
38. Groblewski, T., Maigret, B., Larguier, R., Lombard, C., Bonnafous, J. C., and Marie, J. (1997) *J. Biol. Chem.* **272**, 1822–1826
39. Marie, J., Koch, C., Pruneau, D., Paquet, J. L., Groblewski, T., Larguier, R., Lombard, C., Deslauriers, B., Maigret, B., and Bonnafous, J. C. (1999) *Mol. Pharmacol.* **55**, 92–101
40. Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P., and Clauser, E. (2002) *Trends Endocrinol. Metab.* **13**, 336
41. Smith, J. A., Webb, C., Holford, J., and Burgess, G. M. (1995) *Mol. Pharmacol.* **47**, 525–534
42. Decarie, A., Raymond, P., Gervais, N., Couture, R., and Adam, A. (1996) *Am. J. Physiol.* **271**, H1340–H1347
43. McLean, P. G., Perretti, M., and Ahluwalia, A. (2000) *Cardiovasc. Res.* **48**, 194–210
44. Alvarez, A. L., Delorenzi, A., Santajuliana, D., Finkielman, S., Nahmod, V. E., and Pirola, C. J. (1992) *Clin. Sci. (Colch.)* **82**, 513–519
45. Emanueli, C., Chao, J., Regoli, D., Chao, L., Ni, A., and Madeddu, P. (1999) *Br. J. Pharmacol.* **126**, 1769–1776
46. Decarie, A., Adam, A., and Couture, R. (1996) *Peptides* **17**, 1009–1015
47. Campos, M. M., Souza, G. E., and Calixto, J. B. (1996) *Br. J. Pharmacol.* **117**, 793–798
48. Whalley, E. T., Sampaio, C. A., Fritz, H., and Edwards, G. (1984) *Naunyn Schmiedebergs Arch. Pharmacol.* **328**, 202–206
49. Nwator, I. A., and Whalley, E. T. (1989) *Eur. J. Pharmacol.* **160**, 125–132